PHYLOGENY OF THE RUBIACEAE–RUBIOIDEAE, IN PARTICULAR THE TRIBE RUBIEAE: EVIDENCE FROM A NON-CODING CHLOROPLAST DNA SEQUENCE[1]

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ABSTRACT

A phylogenetic analysis of 39 species of the tribe Rubieae and of 15 taxa belonging to 12 other tribes of Rubiaceae has been performed using the DNA sequence of the chloroplast *atp*B-*rbc*L intergene region. The subfamily Rubioideae may be characterized as a monophylum, i.e., by a characteristic 204-bp deletion, shared by the representative tribes Coccocypseleae, Psychotrieae, Hedyotideae (paraphyletically linked to Spermacoceae), Anthospermeae, Theligoneae, and Paederieae, which, in this order, step-wise approach the advanced Rubieae. This tribe is clearly monophyletic and characterized by an additional 50-bp deletion. Five clades can be recognized within Rubieae, which mostly corroborate, but also partly contradict, traditional groupings (i.e., *Galium* and *Asperula* appear to be of polyphyletic origin); some of these results may have taxonomic implications.

By comparing the nucleotide sequences of a well-defined chloroplast intergene region among different genera and species, we hope to contribute to the reconstruction of the phylogeny of the tribe Rubieae. This tribe belongs to the huge, mostly woody, tropical and subtropical family Rubiaceae, one of the largest of all angiosperm families, with about 637 genera and more than 10,000 species (Mabberley, 1987). In contrast, the Rubieae, containing predominantly perennial to annual herbs with pseudowhorls of leaves and leaflike stipules, and composed of about 13 genera (Ehrendorfer, unpublished), is centered in temperate and tropical-mountain regions. The evolutionary radiation of the Rubieae has resulted in a worldwide distribution, but apparently is relatively recent. A survey of the literature (Muller, 1981) indicates that the first fossil pollen records of the Rubieae are from the Upper Miocene for the genus *Rubia* (Van Campo, 1976) and the Pliocene for the genus *Galium* (Menke, 1976). Because the tribe Rubieae is supposedly of relatively recent origin, instead of the widely used *rbc*L sequence, a non-coding sequence of the chloroplast DNA, the *atp*B-*rbc*L spacer, was chosen. We thought that this sequence, being under lower selective constraints, would exhibit higher variability between the different studied genera and species. This assumption has been proposed by Gielly & Taberlet (1994) for a study of the genus *Gentiana*, using other non-coding cpDNA sequences.

By comparison of this non-coding sequence (Manen et al., 1994), we had presented a phylogenetic analysis of 25 species of the tribe Rubieae, using six tropical genera from other tribes of Rubiaceae as outgroups. In a separate paper (Ehrendorfer et al., 1994) we briefly discussed the relationships among these outgroup tribes and their affinities with the tribe Rubieae. In this paper we extend these data by adding the sequences of 23 more Rubiaceae taxa. This allows a precise and useful delimitation of the subfamily Rubioideae by a very characteristic deletion, as well as, in spite of the addition of many taxa, the confirmation of the previously suggested general traits of the phylogeny of the tribe Rubieae and of the polyphyly of the genera *Galium* and *Asperula*.

MATERIALS AND METHODS

The list of the Rubiaceae taxa studied so far is shown in Table 1. It represents 8 genera, 39 spe-

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Table 1. Sources of cpDNA (fresh leaves) from the Rubiaceae species: 64 populations belonging to 54 taxa. * The numbers correspond to the numbers that appear in the phylogenetic trees (see Figures 1 and 2). ** Collectors: JEA = Jeanmonod, Daniel; MAN = Manen, Jean-François; NAT = Natali, Alessandro; PAL = Palese, Raoul; ROG = Roguet, Didier; THI = Thiébaud, Marc-André; ZEL = Zelweger, Catherine; E = Ehrendorfer, Friedrich; MK = Kiehn, Michael. The number is the collector number; all vouchers have been deposited in the herbaria of Geneva (G) or Universität Wien (WU). JBG + number stands for the living collection number in the Botanical Garden of Geneva. HBV + number stands for the living collection number in the Botanical Garden of Vienna.

No.*	Species	Locality	Voucher information**
Rubieae			
33	*Rubia peregrina* L.	Elba Island	NAT & THI/N56965
11	*R. tinctorum* L.	Geneva, Bot. Gard.	JBG 916690
02	*Sherardia arvensis* L.	Corsica, St. Petrone	JEA & NAT/J5048
05	*Sherardia arvensis* L.	Geneva	NAT & MAN/007
29	*Crucianella angustifolia* L.	Corsica, Francardo	JEA & NAT/J5044
35	*Phuopsis stylosa* (Trin.) Jackson	Geneva, Bot. Gard.	JBG 916798
19	*Asperula laevigata* L.	Elba Island	NAT & THI/s.n.
01	*A. tinctoria* L.	Geneva, Bot. Gard.	JBG 780680
51	*A. hirta* Ramond	Geneva, Bot. Gard.	JBG 814140/0 M
69	*A. chlorantha* Boiss. & Heldr.	Greece, Epirus	E 930413-4401
81	*A. cynanchica* L.	Geneva, Bot. Gard.	JBG 861771/0
82	*A. gussonii* Boiss.	Geneva, Bot. Gard.	JBG 783214/0
77	*A. purpurea* (L.) Ehrend.	Italy, Alpi Apuane	NAT & MAN/011
34	*Cruciata laevipes* Opiz	Corsica, Radicale	JEA, NAT, PAL/J4198
22	*C. glabra* (L.) Ehrend.	Elba Island	NAT & THI/N57761
32	*Valantia muralis* L.	Corsica, Pigno	JEA & NAT/s.n.
12	*Galium mollugo* L.	Corsica, Calvi	JEA, NAT, ZEL/s.n.
03	*G. album* L.	Corsica, Solenzara	JEA, NAT, PAL/s.n.
25	*G. album* L.	Corsica, S. Michelle	JEA & NAT/J4969
13	*G. album* L.	Geneva, Lullier	NAT & MAN/008
30	*G. album* L.	Corsica, Miomo	JEA & NAT/J4935
26	*G. album* L.	Corsica, St. Florent	JEA & NAT/J4963
31	*G. corrudifolium* Vill.	Elba Island	NAT & THI/N56941
23	*G. verum* L.	Geneva, Lullier	NAT & MAN/009
18	*G. lucidum* All.	Elba Island	NAT & THI/N56959
08	*G. lucidum* All.	Corsica, Strette	JEA & NAT/J4964
21	*G. aetnicum* Biv.	Capraia Island	NAT & THI/N57944
09	*G. corsicum* Spreng.	Corsica, Col St. Jean	JEA & NAT/J4931
07	*G. scabrum* L.	Corsica, Porto	JEA & ROG/J4961
20	*G. scabrum* L.	Elba Island	NAT & THI/N56964
16	*G. scabrum* L.	Elba Island	NAT & THI/N57753
17	*G. rotundifolium* L.	Corsica, Loreto di Casinca	JEA & NAT/J4979
06	*G. elongatum* C. Presl	Corsica, St. Florent	JEA & NAT/J4966
14	*G. palustre* L.	Geneva	NAT & MAN/s.n.
04	*G. divaricatum* Lam.	Corsica, Ajaccio	JEA, NAT, ZEL/J3394
10	*G. parisiense* L.	Corsica, Radicale	JEA, NAT, PAL/J4186
24	*G. verrucosum* Hudson	Corsica, Bonifacio	JEA, PAL, ROG/J3980
15	*G. aparine* L.	Corsica, Pietrabugno	JEA & NAT/s.n.
27	*G. aparine* L.	Corsica, St. Petrone	JEA & NAT/J5017
28	*G. aparine* L.	Corsica, Ponte Leccia	JEA & NAT/s.n.
52	*G. baillonii* Brandza	Romania, Prov. Arges	E 890821-3001
54	*G. murale* (L.) All.	Ionian Islands	E 930409-2502
55	*G. intricatum* Margot & Reuter	Ionian Islands	E 930409-2501
70	*G. perralderii* Coss.	Algeria, Kabylie	E 930626-1001
75	*G. rubioides* L.	Geneva, Bot. Gard.	NAT & MAN/013
76	*G. tricornutum* Dandy	Geneva, Bot. Gard.	NAT & MAN/014
79	*G. boreale* L.	Geneva, Bot. Gard.	JBG 814159/0
80	*G. odoratum* (L.) Scop.	Geneva, Versoix	NAT & MAN/016
45	*G. septentrionale* Roem. & Schult. (=*G. boreale* L. ?)	USA, Colorado	NAT & MAN/017

Table 1. Continued.

No.*	Species	Locality	Voucher information**
Paederieae			
53	*Putoria calabrica* (L. f.) DC.	Ionian Islands	E 930416-6201
Theligoneae			
67	*Theligonum cynocrambe* L.	Ionian Islands	E 930416-6201
Anthospermeae			
59	*Coprosma montana* Hillebr.	Hawaiian Islands, Maui	MK-910114-1/1
60	*C. ernodeoides* A. Gray	Hawaiian Islands, Maui	MK-910114-1/2
Spermacoceae			
65	*Spermacoce assurgens* Ruiz & Pavón	Costa Rica, Guanacaste	MK-880317-2/2
Psychotrieae			
42	*Hydnophytum formicarum* Jack	Geneva, Bot. Gard.	NAT & MAN/001
44	*Psychotria bacteriophila* Valet.	Geneva, Bot. Gard.	NAT & MAN/002
Hedyotideae			
41	*Bouvardia glaberrima* Engelm.	Geneva, Bot. Gard.	NAT & MAN/003
47	*Pentas lanceolata* (Forssk.) Deflers	Geneva, Bot. Gard.	NAT & MAN/004
Coccocypseleae			
64	*Coccocypselum* sp.	French-Guayana	HBV RR-91-19
Ophiorrhizeae			
63	*Ophiorrhiza* sp.	Indonesia, Sumatra	HBV RR-89-6
Hillieae			
72	*Hillia valerii* Standl. [=*Cosmibuena valerii* (Standl.) C.M. Taylor]	Costa Rica, Heredia	MK-880331-1/2
Hamelieae			
73	*Hoffmannia refulgens* Hemsl.	Mexico, Oaxaca	HBV RR-91-17
Coffeeae			
37	*Coffea arabica* L.	Geneva, Bot. Gard.	NAT & MAN/005
Pavetteae			
38	*Ixora parviflora* Vahl	Geneva, Bot. Gard.	NAT & MAN/006

cies, and 49 samples for the tribe Rubieae. To evaluate possible infraspecific variations, from several species different populations coming from various regions have been studied, including three different populations of the polymorphic *Galium aparine*, three populations of *Galium scabrum*, two populations of *Galium lucidum*, five populations of *Galium album*, and two populations of *Sherardia arvensis*. When variations (in fact very few) have been detected, the variable taxa have been included in the analysis. To delimit the tribe Rubieae and to test its monophyly, outgroup species, mainly from the subfamily Rubioideae, have been included in the analysis. Altogether, this analysis is based on 64 samples representing 54 species of Rubiaceae. All of these sequences are registered in the EMBL data bank under the accession codes X76457 to X76481 and X81669 to X81690.

Plant material was field-collected, obtained from seed that we grew in the greenhouses of the Geneva Botanical Garden, or taken from the living collections of the Botanical Gardens in Geneva and Vienna. Following the recommendations of Goldblatt et al. (1992), for each analyzed sample a voucher specimen has been prepared and deposited in the Geneva (G) or Vienna University (WU) herbaria.

The sequence we have chosen for this analysis is the intergene sequence located between the *rbc*L and the *atp*B gene of the plastid genome (see Manen et al., 1994, for more details). The *rbc*L and *atp*B genes are on opposite DNA strands. Thus, their intergene sequence contains the promoters of the two genes. We have studied this intergene region in a wide range of dicotyledonous orders and found that the *atp*B gene is regulated by two different promoters, whose functions are

certainly distinct (Manen et al., 1993). The sequenced region comprises also the first 56 *rbc*L codons.

The total aligned DNA matrix is available on request from the authors. It is 1152 sites long, including gaps. A careful analysis of this DNA matrix prompted us to discard from the analysis three small regions. These positions are: (1) The position 706–716, which represents the link between the *rbc*L leader sequence and the *atp*B leader sequence. We have discarded from the analysis this highly variable stretch of adenosine nucleotides. (2) The position 875–878, which is subject to an interesting intramolecular recombination of four nucleotides, GTGA, to its complementary TCAC. The mechanism of this recombination is not known, but a stem-loop structure (favored by the surrounding conserved inverted repeat) is certainly involved. These nucleotide changes are obviously not independent and were treated as a unique event. (3) Following the positions 1138–1140, a CG rich region is susceptible to sequencing errors. We have removed these three sites from our analysis.

Phylogenetic (parsimony) analyses of this DNA matrix were conducted with the PAUP program (version 3.1, Swofford, 1991) on a Quadra 700 Macintosh computer. Only phylogenetically informative characters have been analyzed. Except when indicated in the text, heuristic searches have been conducted with 10 replications of random addition of sequences, TBR branch swapping, and MULPARS options. Because of the size of the matrix, only 100 bootstrap replications have been conducted.

Results

We have found two rather large characteristic deletions: the first one specific to the subfamily Rubioideae (position 496–699), and the second one specific to the tribe Rubieae (position 221–294). The former consists of a large (204 nucleotides long) deletion in the *atp*B leader sequence, the latter is an additional smaller (about 50 nucleotides long) deletion that is not found in the other members of the Rubioideae. Therefore, a quick look at the DNA matrix is sufficient to distinguish Rubieae from other Rubioideae, and Rubioideae from other Rubiaceae.

Table 2 represents a record of the variable and informative sites found in the DNA matrix of all the studied taxa, or of the Rubieae alone. To evaluate the influence of indels, gaps treated as new states (the fifth base), or gaps treated as missing data, are also compared. This shows the large effect of gaps in the analysis, as well as the smaller amount of variability within the Rubieae as compared to the other Rubiaceae studied here.

Table 2. Record of variable and informative sites. Amount of variable and informative sites in the DNA matrix treated with the gap = newstate or the gap = missing option. These values are also calculated for the Rubieae ingroup alone.

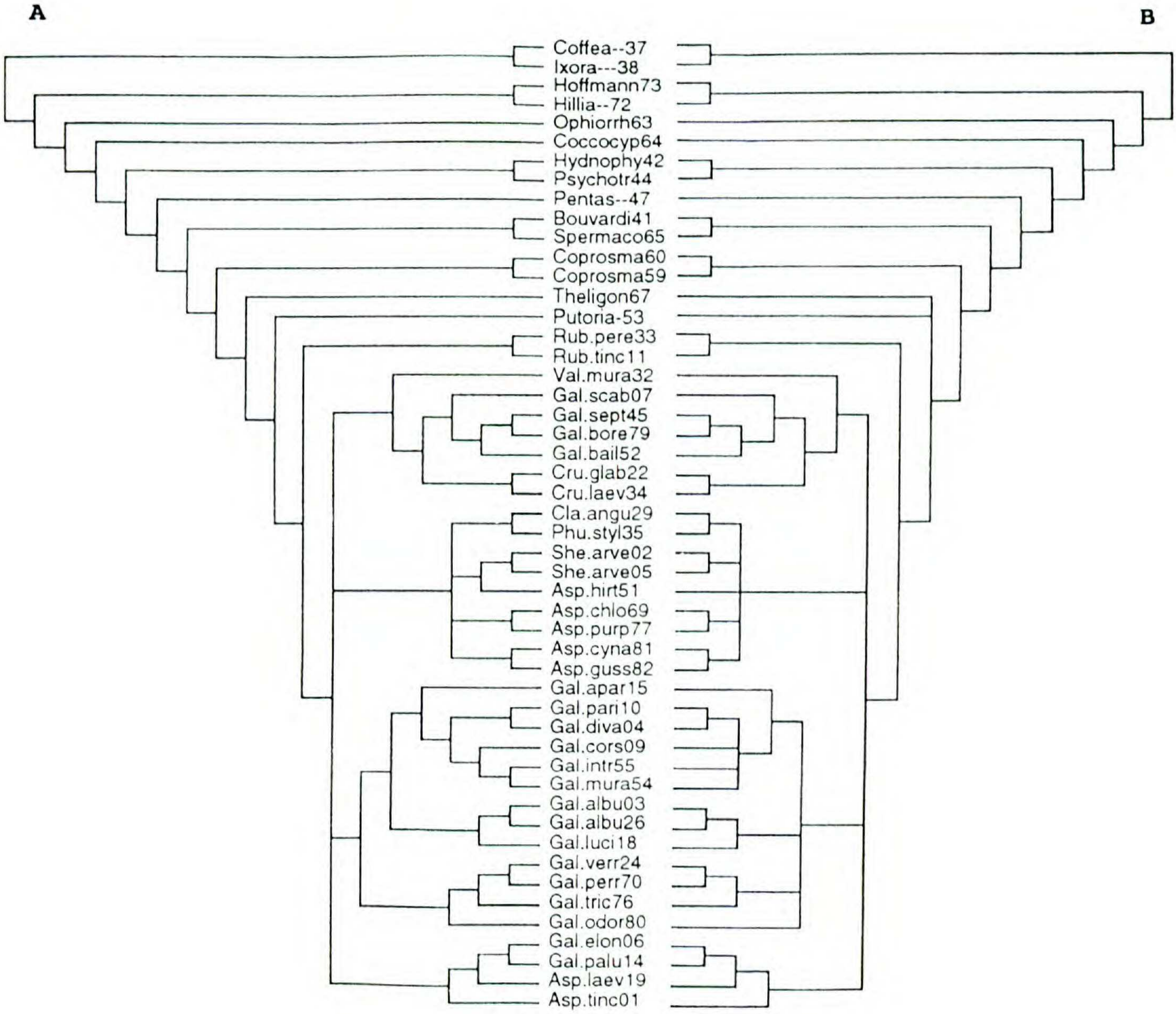
		Variable	Informative
Gap = newstate	All taxa	737	520
	Rubieae	179	95
Gap = missing	All taxa	314	206
	Rubieae	144	68

In order to evaluate the influence of the gaps, two different analyses of the DNA matrix were conducted: one with the option "gap = newstate" (gaps treated as multiple additive independent events), and the other with the option "gap = missing" (Fig. 1). (1) For the "gap = newstate" matrix, we used a heuristic search, with 10 replications of random addition of sequences and the TBR branch swapping option. Only three most parsimonious trees were obtained. Figure 1A shows the strict consensus of these trees. They are 1027 steps long, and the consistency index (C.I.), excluding uninformative sites, is 0.659, which is a rather high value. (2) For the "gap = missing" matrix, we used a heuristic search, without replication. Figure 1B shows the consensus tree of the huge amount (more than 1500) of different most parsimonious trees found (392 steps long, C.I. = 0.643).

The consensus tree obtained with the "gap = missing" option is very similar to the consensus tree obtained with the "gap = newstate" option. In spite of minor and non-contradictory dissimilarities, the general topology is conserved. This is encouraging in view of the two very different matrices used, and strongly supports the topology of the phylogenetic tree obtained.

Figure 2 shows a cladogram of one of the three most parsimonious trees obtained with the "gap = newstate" matrix. We chose to present it because it presents the same topology as the consensus tree of Figure 1, while the two others have little more information not sustained by the consensus. The bootstrap values are indicated on the cladogram of Figure 2.

For more details inside the Rubieae, an analysis including only *Theligonum*, *Putoria*, and all the Rubieae species is presented in Figure 3 as a phylogram (in which the branch lengths are propor-




```
Gap = newstate

Consensus of 3 trees
Tree length = 1027
CI = 0.659
```

```
Gap = missing

Consensus of >1500 trees
Tree length = 392
CI = 0.643
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Figure 1. Comparison of the cladograms obtained either with the gap = newstate or the gap = missing option.— A. Strict consensus tree obtained with the option gap = newstate. Three most parsimonious trees were produced.— B. Strict consensus tree obtained with the option gap = missing. More than 1500 most parsimonious trees were produced. For the Rubieae ingroup the species names are indicated by the three first letters of the genus and the first four letters of the species, except for *Crucianella* = Cla. For the other species, the first eight letters of the corresponding genus are indicated. The numbers following the species names correspond to those indicated in Table 1.

tional to the number of steps). We used a heuristic search (gap = newstates option), with 10 replications of random addition of sequences and the TBR branch swapping option. Only three most parsimonious trees were obtained (232 steps, C.I. = 0.694). As explained above, we chose to present the tree as having the same topology as the consensus.

Discussion

As detailed above, we found a strongly supported separation between a first group of taxa (*Coffea*, *Ixora*, *Hoffmannia*, *Hillia*, *Ophiorrhiza*) from different subfamilies and a second group with the remaining taxa all clearly belonging to the subfamily Rubioideae. These groups are distinguished by 192 steps, which are mostly due to the 204-nu-

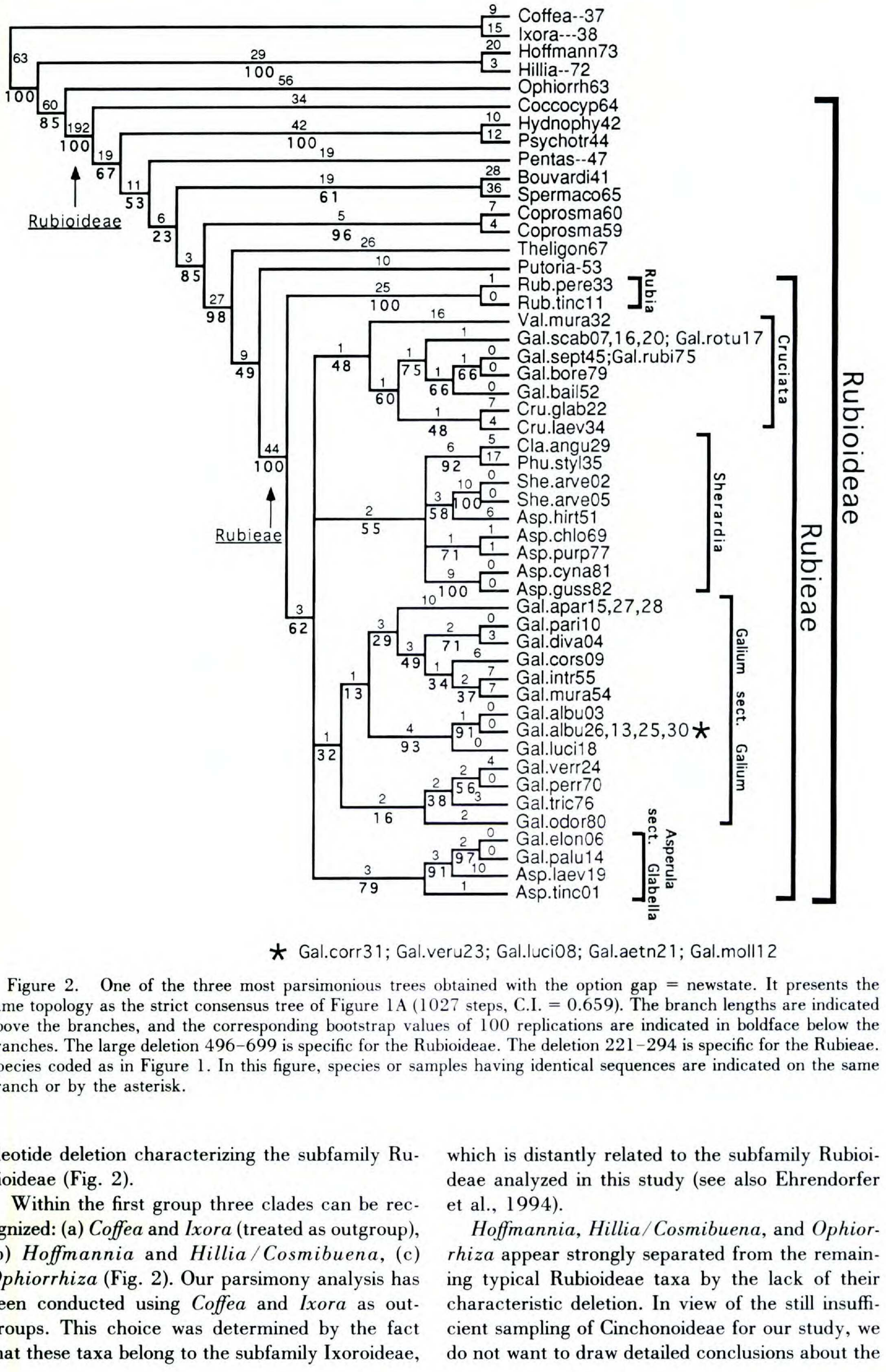

Figure 2. One of the three most parsimonious trees obtained with the option gap = newstate. It presents the same topology as the strict consensus tree of Figure 1A (1027 steps, C.I. = 0.659). The branch lengths are indicated above the branches, and the corresponding bootstrap values of 100 replications are indicated in boldface below the branches. The large deletion 496–699 is specific for the Rubioideae. The deletion 221–294 is specific for the Rubieae. Species coded as in Figure 1. In this figure, species or samples having identical sequences are indicated on the same branch or by the asterisk.

cleotide deletion characterizing the subfamily Rubioideae (Fig. 2).

Within the first group three clades can be recognized: (a) *Coffea* and *Ixora* (treated as outgroup), (b) *Hoffmannia* and *Hillia/Cosmibuena*, (c) *Ophiorrhiza* (Fig. 2). Our parsimony analysis has been conducted using *Coffea* and *Ixora* as outgroups. This choice was determined by the fact that these taxa belong to the subfamily Ixoroideae, which is distantly related to the subfamily Rubioideae analyzed in this study (see also Ehrendorfer et al., 1994).

Hoffmannia, *Hillia/Cosmibuena*, and *Ophiorrhiza* appear strongly separated from the remaining typical Rubioideae taxa by the lack of their characteristic deletion. In view of the still insufficient sampling of Cinchonoideae for our study, we do not want to draw detailed conclusions about the

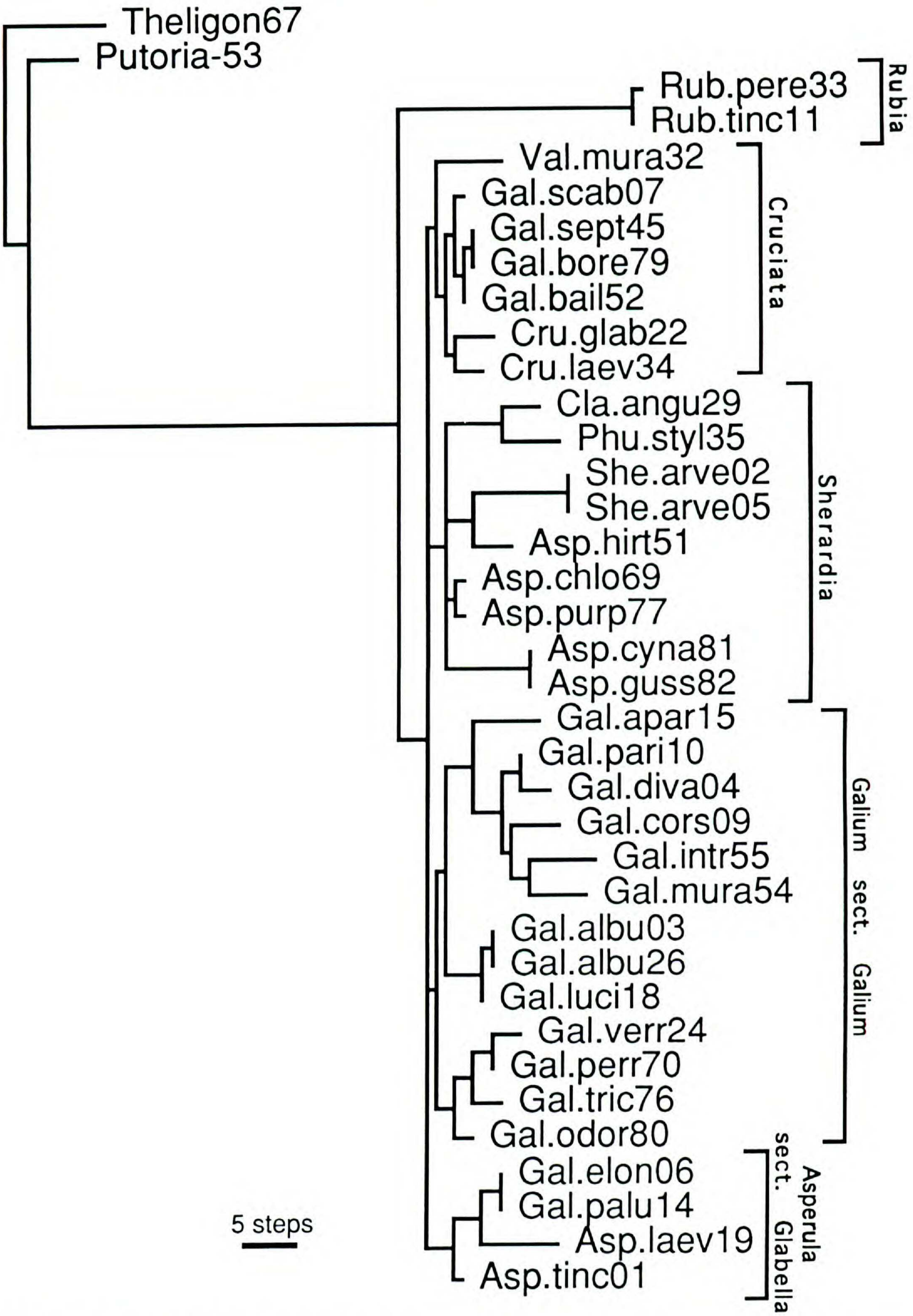

Figure 3. Phylogram (PAUP) including only *Theligonum*, *Putoria*, and the Rubieae species. One of the three most parsimonious trees obtained with the option gap = newstate (232 steps, C.I. = 0.694). Species coded as in Figure 1. For clarity, species or samples having identical sequences are not indicated, and can be followed from Figure 2.

systematics of these taxa. Nevertheless, while our placement of *Hillia valerii* (= *Cosmibuena valerii*) outside the Rubioideae corroborates the most recent works on the tribe Hillieae (Robbrecht, 1988; Andersson & Persson, 1991; Andersson, 1993), the position of *Hoffmannia* and *Ophiorrhiza* is not in accordance with the data of most of the authors. In fact, *Hoffmannia* (represented here by *H. refulgens* from Mexico) belongs to the tribe Hamelieae, which has been included in the subfamily Rubioideae because of the occurrence of raphides (Bremekamp, 1966; Verdcourt, 1958; Robbrecht, 1988). However, the position of Hamelieae quite distant from the remaining members of Rubioideae is in accordance with restriction cpDNA data (Bremer & Jansen, 1991). In a more recent contribution, Bremer & Struwe (1992) affirmed that both molecular and morphological analyses support a position of the tribe Hamelieae outside the Rubioideae. The systematic position of *Ophiorrhiza* has always been very controversial. The views of Verdcourt (1958), Darwin (1976), and Robbrecht (1988), which included this genus in the subfamily Rubioideae close to the Hedyotideae, seem to be contradicted by our molecular data, because *Ophiorrhiza* appears clearly separated from the remaining Rubioideae taxa (including the Hedyotideae genera *Bouvardia* and *Pentas*) by the lack of their characteristic deletion.

The second group of taxa includes unquestionable members of the Rubioideae that share the large 204-nucleotide deletion in the *rbc*L-*atp*B intergene region. This drastic evolutionary change characterizes an obvious monophyly. Our study clearly supports the more modern concept (Robbrecht, 1988) that the presence or absence of raphides should not be overemphasized, as in the system of Verdcourt (1958). Following our molecular data, Rubioideae apparently always have raphides, but there are taxa (*Hillia/Cosmibuena*, *Ophiorrhiza*, *Hoffmannia*) outside this clearly cpDNA-defined subfamily where this character is also present. However, our results are only partially in agreement with the hypothesis presented by Robbrecht (1993) that Rubioideae should essentially be restricted to tribes with uniovulate locules and that the multiovulate species placed in the Rubioideae show more affinity to Cinchonoideae. In fact, our molecular data demonstrate that *Coccocypselum* and some genera of Hedyotideae with multiovulate locules are clearly members of Rubioideae. The independent molecular data from Bremer et al. (1995, this issue) corroborate this view. Our cladogram suggests that the derivation of uniovulate locules occurred several times in parallel from a multiovulate basal phylogenetic line within Rubioideae. This conclusion is in line with Robbrecht's (1993) view that most of the characters used to distinguish major groups in the family evidently have evolved in a parallel fashion several times.

In the following paragraphs we will discuss the clades separated by our molecular analysis within unquestionable Rubioideae (see Fig. 2). Obviously, the fact that only 10 of the 16 tribes that Robbrecht (1988) included in Rubioideae have been analyzed in our study makes our conclusions still preliminary.

Coccocypselum is generally recognized to form a monotypic tribe, Coccocypseleae, placed in the subfamily Rubioideae (Verdcourt, 1958; Bremekamp, 1966; Robbrecht, 1988). This is supported by our data as well as by the results of cpDNA restriction site mapping (Bremer & Jansen, 1991; Bremer & Struwe, 1992). Nevertheless, we cannot confirm the view of these authors of placing *Coccocypselum* between the Psychotrieae and the Hedyotideae clades. According to our data, it constitutes a basal tribe within Rubioideae with multiovulate locules and thick-walled exotestal cells. This is now supported by the most recent work of Bremer et al. (1995, this issue) using *rbc*L sequences.

The Psychotrieae, with consistently uniovulate locules, evidently constitute a clade of the Rubioideae that separated early, as is clearly shown by our intergene sequences as well as by the restriction and *rbc*L data (Bremer & Jansen, 1991; Bremer et al., 1995). The Hedyotideae, clearly belonging to Rubioideae, are another tribe with multiovulate locules, but mostly advanced parenchyma-like exotesta. Whereas *Pentas* was always placed here, *Bouvardia* has been classified in the tribe Cinchoneae (subfamily Cinchonoideae) by Schumann (1891) and, tentatively, by Robbrecht (1988), in contrast to Verdcourt (1958) and Bremekamp (1966), who transferred it because of its raphides to Hedyotideae; the same placement has been suggested by the cladistic analysis of Andersssson & Persson (1991). While all available cpDNA data clearly support this latter view, they raise the problem of a para- or even polyphyletic nature of the Hedyotideae. Whereas earlier suggestions about closer affinities of *Pentas* with Anthospermeae than with *Bouvardia* (Bremer & Jansen, 1991) have been dropped recently (Bremer et al., 1995), our and Bremer's molecular data clearly converge in suggesting that Spermacoceae share ancestry with Hedyotideae. Our cladogram (Fig. 2) shows *Bouvardia* much closer to *Spermacoce* than to *Pentas*,

and Bremer et al. (1995) demonstrate the same from independent data for the Spermacoceae genus *Richardia*. Close relationships between Hedyotideae and Spermacoceae are also suggested by karyological data (Kiehn, 1986, 1995 this issue) and from studies on the genus *Otiophora* (Igersheim & Rohrhofer, 1993). All this supports the idea that the Spermacoceae, with their one-seeded fruit locules, are derived from multi-seeded Hedyotideae-like ancestors.

Our cpDNA intergene data (see Fig. 2) suggest that the remaining tribes of Rubioideae studied here (i.e., Anthospermeae, Theligoneae, Paederieae, and Rubieae) represent a monophylum, in which the character of uniovulate ovary locules has become fixed. The most recent information based on restriction and *rbc*L analyses (Bremer et al., 1995) indicate at least a close proximity of these tribes.

The tribe Anthospermeae, represented here by two species of *Coprosma*, forms a well-separated clade as already suggested by Bremer & Jansen (1991) and Bremer et al. (1995). The monophyly of the Anthospermeae is also supported by several morphological characters (Puff, 1986).

The small tribe Theligoneae, represented here by the Mediterranean *Theligonum cynocrambe*, clearly belongs to the subfamily Rubioideae and appears relatively close to the Paederieae and Rubieae (bootstrap value 98%). The Theligonaceae have been formerly associated with Haloragaceae, Hippuridaceae, and Portulacaceae; only recently, Wunderlich (1971) has suggested an affinity with the Rubiaceae. Our molecular data, together with the recent independent findings of Bremer et al. (1995), now give definite evidence that *Theligonum* belongs to the Rubiaceae/Rubioideae.

Putoria calabrica, the only taxon of the tribe Paederieae analyzed, is the closest to the Rubieae among the taxa considered in our study. This is not surprising, as this Mediterranean genus exhibits obvious morphological affinities (flowers in fascicles, corolla infundibuliform) with several more plesiomorphic taxa of Rubieae.

Finally, the tribe Rubieae is clearly monophyletic according to the available molecular data from 8 genera (out of the 13 to be included in the tribe) and 39 species. This is strongly supported by 44 steps, which are mostly due to a specific 50-nucleotide deletion that does not exist in the remaining Rubioideae (Fig. 3).

The sequence analysis of 14 additional species belonging to the Rubieae has not greatly changed the topology of the tribal cladogram presented previously and based on 25 taxa (Manen et al., 1994). In fact, again five clades can actually be distinguished within the tribe. In our previous work (Manen et al., 1994) these five clades were well separated by all the different methods used for the phylogenetic analysis of the available DNA data.

(1) *RUBIA* CLADE

Our phylogenetic analysis clearly shows the genus *Rubia* as a sister group of the other Rubieae taxa. The separation from the other clades is clear whatever method or matrix is used (Manen et al., 1994), and is supported by a 62% bootstrap value.

In our phylogenetic reconstruction *Rubia* appears to be derived from the ancestor of the Rubieae, while classical treatments based on morphological data usually have considered this genus to be advanced. Nevertheless, the occurrence of plesiomorphic characters in all or part of *Rubia*, e.g., woody growth forms, leaves and leaflike stipules in whorls of four, 5-lobed and sometimes even funnel-shaped corollas, and fleshy drupaceous fruits, suggests a basal position in the tribe.

(2) *SHERARDIA* CLADE

This clade comprises *Sherardia arvensis*, the group of *Crucianella* (abbreviated in the cladogram as Cla; Cru is *Cruciata*) and *Phuopsis*, and the majority of the species studied from the heterogeneous genus *Asperula*. It is not surprising to find *A. cynanchica* and *A. gussonei* from section *Cynanchicae* closely associated. Sequence data support the placement of *A. purpurea* (with rotate flowers and formerly classified as *Galium purpureum*) and *A. chlorantha* (with a long corolla tube) as related in section *Thliphtisa* (Ehrendorfer & Krendl, 1976). *Asperula hirta* (sect. *Hexaphylla*) exhibits some affinities with *Sherardia*, which in turn is also allied to the closely related genera *Crucianella* and *Phuopsis*. Even if the sequence links between all these different subgroups of the clade are not very strong, their inclusion in a monophylum is not surprising in view of several morphological similarities and suspected relationships (see, e.g., Richard, 1829). In our previous publication (Manen et al., 1994) we were surprised to find *Sherardia* far from the *Asperula* species analyzed; but, as we expected, a broader sampling of the heterogeneous genus *Asperula* now has closed the intergene cpDNA gap between it and *Sherardia*.

(3) *ASPERULA* SECT. *GLABELLA* CLADE

The inclusion of several additional species from the genera *Galium* and *Asperula* in our molecular analysis has absolutely not changed the topology

of this clade, which represents one of the most interesting results presented in our previous publication (Manen et al., 1994). On first sight, it was surprising to find that the closely related species *Galium palustre* and *G. elongatum* exhibit much stronger cpDNA affinities with *Asperula* sect. *Glabella* than with the other *Galium* species studied. These two perennial species of swamp and marsh habitats belong to the small section *Aparinoides* (Ehrendorfer & Krendl, 1976). This section is characterized by several aberrant features, such as plants usually turning blackish when dry, obtuse (and not acute) leaves, slightly campanulate flowers, globose (and not ovoid) mericarps, and the chromosome base numbers $x = 12$ (and not 11 as in nearly all other members of *Galium*). All this has made the systematic position of section *Aparinoides* uncertain, and has prompted one of us (F. E.) to suspect an affinity with *Asperula* sect. *Glabella*, where the differential characters mentioned reappear. This suspicion is remarkably supported by the present molecular data and should lead us to reconsider the taxonomic position of section *Aparinoides*, surely separated from the other *Galium* species.

(4) *CRUCIATA* CLADE

The coherence of *Valantia* and *Cruciata* (abbreviated as Cru in the cladogram) in respect to our sequence data is not surprising, since strong morphological and karyological relationships between these two small genera from the Near East, the Mediterranean, and temperate western Eurasia have already been demonstrated (Ehrendorfer, 1971).

We find in the same clade *Cruciata* and *Valantia* together with members of *Galium* sect. *Platygalium*, whereas the latter appears well separated from the taxa arranged inside the *Galium* clade. This justifies our concept of a distinct *Cruciata* clade (Manen et al., 1994), now broadened by the analysis of additional species. This clade is also supported by some important morphological characters, such as pseudowhorls of only two leaves and two leaflike stipules, each often with three parallel veins. The terminal inflorescence (thyrsus) and the hermaphrodite flowers in (most species of) *Galium* sect. *Platygalium* are plesiomorphic features, whereas *Cruciata* and *Valantia* are more advanced with respect to their apically vegetative inflorescence (with lateral cymes) and their andromonoecious flowers.

Galium sect. *Platygalium* comprises a large and diverse group of species with worldwide distribution that is still difficult to define. The sequence analyses of the species studied so far reflect some of its subgroups: *G. scabrum* and *G. rotundifolium* are members of a Western Eurasian/African $2x$-aggregate, whereas *G. baillonii* ($2x$, Carpathian Mts.), *G. boreale* and *G. septentrionale* (polyploids, Eurasia and North America, probably conspecific), and *G. rubioides* (high polyploid, Eurasia) are members of a polymorphic Northern Hemisphere polyploid complex.

The molecular data show that *Valantia* is the sister group to a clade containing *Cruciata* and *Galium* sect. *Platygalium*. This is in contrast with the morphological views (Ehrendorfer, 1971; Ehrendorfer & Krendl, 1976), which suggest that *Valantia* and *Cruciata* are the most closely related taxa and are more advanced than *Galium* sect. *Platygalium*.

(5) *GALIUM* SECT. *GALIUM* CLADE

Monophyly is suggested by our molecular data for this clade, represented here by the perennial sections *Galium*, *Leiogalium*, *Leptogalium*, *Hylaea*, and the annual *Kolgyda*. It is well separated from *Galium* sect. *Platygalium* and section *Aparinoides*. However, its internal relationships are still insufficiently understood. The core of the *Galium* clade, with the two closely related sections *Leiogalium* (with *G. mollugo*, *G. album*, *G. corrudifolium*, *G. lucidum*, *G. aetnicum*) and *Galium* (with *G. verum*), exhibits practically no sequence differences. This is not surprising in view of extensive hybridization documented within and between these sections (e.g., *G. verum* × *G. album*).

Since Linnaeus's (1753) *Species Plantarum* the taxon *Galium odoratum* (sect. *Hylaea*) has been regarded as a core species of the genus *Asperula*, differentiated originally only on the basis of its infundibuliform corolla shape. Nevertheless, there are several typical *Galium* species from Asia with rotate corollas that closely correspond in all other characters with "*Asperula*" *odorata*, e.g., *G. asperuloides*, *G. hofmeisteri*, *G. japonicum*. This, and the lack of morphological links with other, unquestionable members of *Asperula*, has prompted the taxonomic transfer of this and other taxa wrongly placed in *Asperula* to *Galium* (Ehrendorfer, 1948: 232 f.; 1958: 353; Ehrendorfer & Krendl, 1976). This transfer is now convincingly backed by our molecular data.

The grouping inside this clade is not sufficiently supported by the bootstrap and has to be regarded as provisional. *Galium odoratum* (sect. *Hylaea*) and *G. perralderii* (taxonomic position uncertain),

both perennials, may be related and clearly occupy a more basal position within the *Galium* clade. *Galium corsicum* belongs to the short-lived perennials of section *Leptogalium*. All the remaining species studied so far are annuals and have been placed in section *Kolgyda*. Morphologically, however, they form rather distinct subgroups: *G. tricornutum* and *G. verrucosum*; *G. aparine*; *G. parisiense* and *G. divaricatum*; *G. intricatum* and (morphologically highly apomorphic) *G. murale*. Available sequences indicate complex interrelationships and suggest a polyphyletic origin of the section *Kolgyda*.

Conclusions

The results of our molecular study give clear evidence for the monophyletic origin of the tribe Rubieae, at least according to the data from the 8 genera and 39 species that have been analyzed. The Rubieae could have originated from a (sub)tropical ancestor in common with Paederieae in the course of adaptation to temperate regions. Future research could establish the precise geographical location and the age of this ancestor. Further interesting questions will concern the ecophysiological adaptation underlying its change from tropical to temperate environments, and its worldwide migration routes. Similar trends have occurred repeatedly in angiosperm evolution, and are therefore of general relevance.

Within the tribe Rubieae, the 39 species studied so far constitute 5 groups, confirming our previous results (Manen et al., 1994). The obtained data are often in agreement with classical taxonomic views; moreover, in some cases the molecular data strongly support some suspected affinities among systematically distant taxa.

The disparities between the traditional morphological data and our molecular data could be caused by procedural problems, such as equating overall similarity with phylogenetic relationships (Sytsma, 1990). Such discrepancies may also be caused by biological phenomena frequent in Rubieae: unequal rates of morphological evolution can obscure phylogenetic relationships and, moreover, hybridization and polyploidy result in reticulate evolution not resolved by chloroplast sequences.

Our phylogenetic information on the tribes outside Rubieae is obviously still preliminary. However, the discovery of the important deletion apparently specific for the subfamily Rubioideae opens some unexpected opportunities for a rapid and effective delimitation and phylogenetic reconstruction of this subfamily.

This large deletion affects one of the two *atp*B promoters. The deletion of the distal *atp*B promoter in the Rubioideae is a unique feature in the large range of the dicots so far tested (Manen et al., 1993). It would be interesting to look at the biological (metabolic) significance of its sudden disappearance. Since the two *atp*B promoters are suspected to have a specific biological function in the regulation of the expression of the ATP synthase (coupling factor, a key component of the photosynthetic apparatus), the suppression of one of them might have some physiological implications. Could this be linked to the capacity of this subfamily to produce several herbaceous lines, often adapted as colonizers and expanding into xeric and temperate environments?

The results of our phylogenetic study of the Rubieae/Rubioideae, based on sequence data for a chloroplast intergene region, show the great taxonomic potential of this new method. This cpDNA non-coding region shows large amounts of variability from the intrageneric level and upward, offering an effective tool for a phylogenetic approach to the delimitation and relationships of genera, tribes and subfamilies. Nevertheless, these molecular studies will obviously have to be combined with more traditional biosystematic approaches and other molecular methods, since only multidisciplinary cooperation will result in real progress concerning our systematic and phylogenetic understanding of this enormous family.

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